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Fluctuations of Hepatitis C Virus Load Are not Related to Amino Acid Substitutions in Hypervariable Region 1 and Interferon Sensitivity Determining Region

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Hepatitis C virus (HCV) load is one of the most important predictive factors of response to interferon treatment. However, little is known about the mode and determinants of viremia. The mode of viremia was investigated in 78 patients with chronic HCV genotype 1b infection during 1–2 years follow up. Virus load, determined by a branched chain DNA amplification assay, was stable in 73 of 78 (93.6%) patients, whereas 5 (6.4%) showed marked fluctuation (from undetectable level to more than 10 Meq/ml) in viral titer. To study the mechanisms mediating fluctuations in viral titer, amino acid sequences of two regions were examined; hypervariable region (HVR) 1 and the interferon sensitivity determining region (ISDR). Multiple amino acid substitutions were observed in HVR 1 but no relationship was evident between substitutions and virus titers. In contrast, no amino acid substitutions were observed in the ISDR in any patients with stable virus titer during a follow-up period of 12–24 months (7–24 samples) or in one patient who was observed for 15 years. Interestingly, multiple amino acid substitutions in the ISDR appeared in only two of the five patients with marked titer fluctuation, when the virus decreased markedly. Alanine aminotransferase levels in these five patients correlated with viral load. The data suggest that amino acid substitutions in HVR1 and ISDR are not essential for changes in viral titer. Possible mechanisms of fluctuations of viral titer and amino acid substitutions in the ISDR accompanying marked reductions in viral load are discussed. ***J. Med. Virol. 58:247–255, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: interferon therapy; HCV hepatitis; viral titer; interferon sensitivity determining region; hypervariable region

INTRODUCTION

Hepatitis C virus (HCV) is now the major etiologic agent responsible for post—transfusion and sporadic non-A, non-B hepatitis. Chronic infection is common among patients infected with HCV, and is associated with liver cirrhosis and liver cancer in at least 20% and 1–2% of cases, respectively [Dienstag, 1983; Koretz et al., 1985; Kiyosawa et al., 1990]. Interferon is currently the only drug that induces viral clearance and marked biochemical and histological improvement [Chayama et al., 1991; Shindo et al., 1992]. However, only a proportion of treated patients show complete eradication of the virus, whereas the remaining patients often exhibit exacerbation of the disease process [Davis et al., 1989; Di Bisceglie et al., 1989].

Pretreatment viral load is one of the most important predictive factors for a response to therapy and eradication of the virus [Yoshioka et al., 1992; Matsumoto et al., 1994; Tsubota et al., 1994]. The virus is often eliminated in patients with low virus titer whereas such response is seen only occasionally in patients with high titers. Interestingly, Garson et al. [1992] reported that viral titers returned to almost pretreatment levels af-

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ter cessation of interferon therapy in those patients in whom therapy failed to eradicate the virus. Thus, it is likely that there is transient reduction of viral load in each patient. In a recent study, Nguyen et al. [1996] demonstrated that viral titers observed over periods of less than 3 months in untreated patients remained stable, with only little changes in magnitude (less than three—fold) throughout the observation period. Little is known, however, about the typical form of viremia over long—term observation or the exact factors that determine viral load.

Amino acid substitutions in some portions of the viral protein may be related to viral load. One such domain of the viral protein is the hypervariable region 1 (HVR1). HVR1 is a part of the E2/NS1 region, which is thought to form envelope proteins and is the target of neutralizing antibodies. Production of such antibodies may reduce the viral load. In a previous study by Kao et al. [1995], acute exacerbations of hepatitis and abrupt increases in virus titer coincided with multiple amino acid substitutions in HVR1. However, it is not known whether viral genetic drift actually correlates with viral titer.

The interferon sensitivity determining region (ISDR) also correlates with viral titer [Enomoto et al., 1995, 1996; Chayama et al., 1997]; patients with high viral titers often harbor a virus with a few or no substitutions in the ISDR. In contrast, multiple amino acid substitutions in the ISDR are detected in patients with low viral titers. Little is known, however, about the temporal association between amino acid substitutions and changes in viral titer.

In the present study, viral titers were examined in 78 patients with chronic (>1 year) HCV genotype 1b infection. Five patients who exhibited a marked fluctuation of viral titer were identified and the amino acid sequences of HVR1 and the ISDR of HCV RNA extracted from these patients were examined. In addition, the amino acid sequences of these two regions were determined in serum samples obtained from one patient who had exhibited stable alanine amino transferase (ALT) levels for more than 15 years.

MATERIALS AND METHODS

Patients and Serum Samples

Serum samples were obtained from 78 consecutive adult Japanese patients with chronic HCV genotype 1b infection (determined by polymerase chain reaction [PCR] using a genotype—specific primer set [Hashimoto et al., 1996]. Patients were followed up for more than 1 year without any antiviral or immunosuppressive therapy between December 1994 and April 1997. Patients who were treated with interferon within 6 months before observation were excluded from the present study. We also excluded patients who were positive for HBs antigen. Serum samples, collected monthly or every 2 months, were used for routine liver function tests. Aliquots of serum samples were frozen and stored at –80°C until HCV RNA quantification and nucleotide sequence analysis. Serum samples obtained from one patient who was followed up for 15 years with almost normal ALT values were also analyzed. Informed consent was obtained from all participants prior to commencement of the study.

HCV RNA Quantification

RNA quantitation was carried out within 4 days of serum sampling using the above serum aliquots. Serum HCV RNA was measured by the first generation branched DNA amplification (bDNA) assay (Quantiplex™, Chiron Corp, Emeryville, CA) according to the instructions provided by the manufacturer. The results were expressed as millions of genomic equivalents per milliliter (Meq/ml). The lower and upper limits of the assay were 0.5 and 40 Meq/ml, respectively.

Nucleotide and Amino Acid Sequence Analysis of HVR1 and ISDR

Because it was known that HVR1 has many mutations, its nucleotide sequences were determined by the dideoxy method after cloning PCR—amplified DNA fragments into pUC18 or pBluescript (Stratagene, CA) as described previously [Chayama et al., 1995]. Ten clones from each serum sample were sequenced.

Sequences of ISDR, which was known as the conservative region, were determined by direct sequencing using PCR products as templates, as described previously [Chayama et al., 1997]. Cloning and sequencing of three clones of ISDR were also detected on five patients who showed marked fluctuation of the virus titer.

RESULTS

Stability of HCV Titer

Seventy—three (93.6%) of the 78 patients with stable viral titers and only 5 patients (6.4%) had a marked fluctuation (from less than 0.5 Meq/ml to more than 10 Meq/ml) in virus titer throughout a 2—year follow—up period. The former group could be divided into three categories according to HCV titer: the high titer group in whom >80% of bDNA values were >10 Meq/ml (n = 19 [24.4%], Fig. 1a), low titer group with >80% of DNA titers were <0.5 Meq/ml (n = 9 [11.5%], Fig. 1c), and intermediate group consisting of other patients (n = 45 [57.7%], Fig. 1b). The bDNA values in most patients classified as intermediate ranged from 1 to 10 Meq/ml (Fig. 1b). Of the 5 patients with marked fluctuation of virus titer (fluctuation group, Fig. 1d), 4 showed such fluctuation throughout the period of observation, but 1 patient exhibited a persistently high titer in the latter half of the observation period only.

The degree of fluctuation of the virus titer (including the above five patients) is shown in Figure 1 as "fold—change" (median values), the ratio between the highest and lowest HCV titer [Nguyen et al., 1996]. The variability in the five patients with fluctuating levels is clearly evident and easily distinguishable from those of the other three groups.

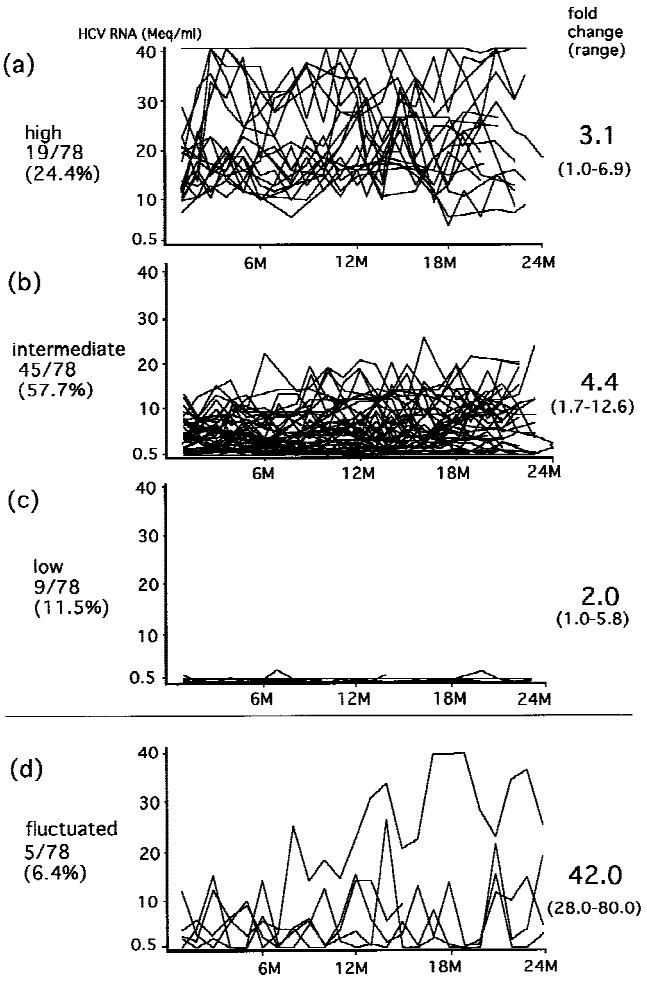


Fig. 1. Serial changes in hepatitis C virus (HCV) titer measured by bDNA assay. **(a)** High titer group; Patients in whom the titer in >80% of serum samples was >10 Meq/ml. **(b)** Intermediate titer group; Patients in whom the titer in >80% of serum samples was <1 Meq/ml. **(c)** Low titer group; The remaining patients, except those shown in (d). **(d)** Fluctuated titer group; Patients who showed marked fluctuations in HCV titer (from <0.5 Meq/ml to >10 Meq/ml). The median values of fold changes and ranges of these titers are shown on the right side of the panel.

Relationship Between Virus Titer and Amino Acid Sequence Substitutions in HVR1 and ISDR

The amino acid sequences of HVR1 and ISDR were examined in HCV obtained from the five patients of the fluctuation group in Figure 1. The clinical course in these five patients (patients A–E) and amino acid sequences of HVR1 and ISDR are shown in each of the sections of Figure 2. Multiple amino acid substitutions were noted in HVR1 in four of the five patients (Figs. 2A–D), but they did not correlate with viral titer or ALT levels. In contrast, multiple amino acid substitutions were observed in ISDR in only two of the five patients (Figs. 2B, D) and only when virus titers were markedly low. Virus titers in the remaining three patients fluctuated markedly without amino acid substitutions in ISDR (Figs. 2A, C, and E).

Correlation Between HCV Titer and ALT Level

As shown in Figure 2, changes in HCV titer tended to correlate with those of ALT activity in all patients of the fluctuation titer group, suggesting that fluctuation of the viral titer was related to hepatocellular damage. In contrast, there was no such correlation in the remaining 73 patients who showed stable viral titer (data not shown).

Stability of Amino Acid Sequence of ISDR During Follow—Up

The amino acid sequences of ISDR were also examined in 34 of 73 patients with no fluctuation of HCV titer. The high titer group indicated 0 or only one amino acid substitution. The intermediate group also indicated 0 or only one substitution and two samples showed two substitutions. The patients belonging to low titer group indicated each 1, 3, 6, and 7 amino acid substitutions, more substitutions than other groups. Direct sequence analysis showed no amino acid substitutions in all samples except one patient (No. 30) during 14–24 months follow up (2–24 samples) (Fig. 3).

Amino acid sequences of the ISDR of the only patient (patient No. 30 in Fig. 3) with amino acid substitutions were further analyzed by cloning and sequencing (Fig. 4). Quasispecies of ISDR variants existed in this patient, as shown in Figure 4c. Amino acid substitutions detected by direct sequencing were due to fluctuations of quasispecies.

Figure 5 shows the amino acid sequences of the HVR1 and ISDR of a patient who was followed up for 15 years. Multiple amino acid substitutions in the HVR1 appeared during the 15-year follow up (Fig. 5b). In contrast, amino acid sequences of the ISDR in serum samples obtained between 1976 and 1991 were completely identical (Fig. 5a). Amino acid sequences of the ISDR were thus stable during short- and long-term follow up in patients who maintained a constant virus load.

DISCUSSION

This study showed that HCV titers are stable in most patients with chronic HCV infection during a period of up to 2 years. These findings are consistent with those of Nguyen et al. [1996], who showed stable diurnal, daily, weekly, and monthly HCV titers, measured by bDNA probe assay, in patients with chronic HCV infection. These findings suggest that there could be some limitations in each infected patient that restrict virus replication and that such conditions are stable in most patients. However, a small proportion of patients (5/78, 6.4%) was identified in whom marked fluctuations in HCV titer were noted. Such patients were not evidently observed in the previous study of Nguyen et al., [1996], probably because of the shorter period of observation and smaller number of patients studied.

Amino acid sequences of HCV in our five patients with marked fluctuations of viral titer were then analyzed during peaks and nadirs of HCV titer levels, in an

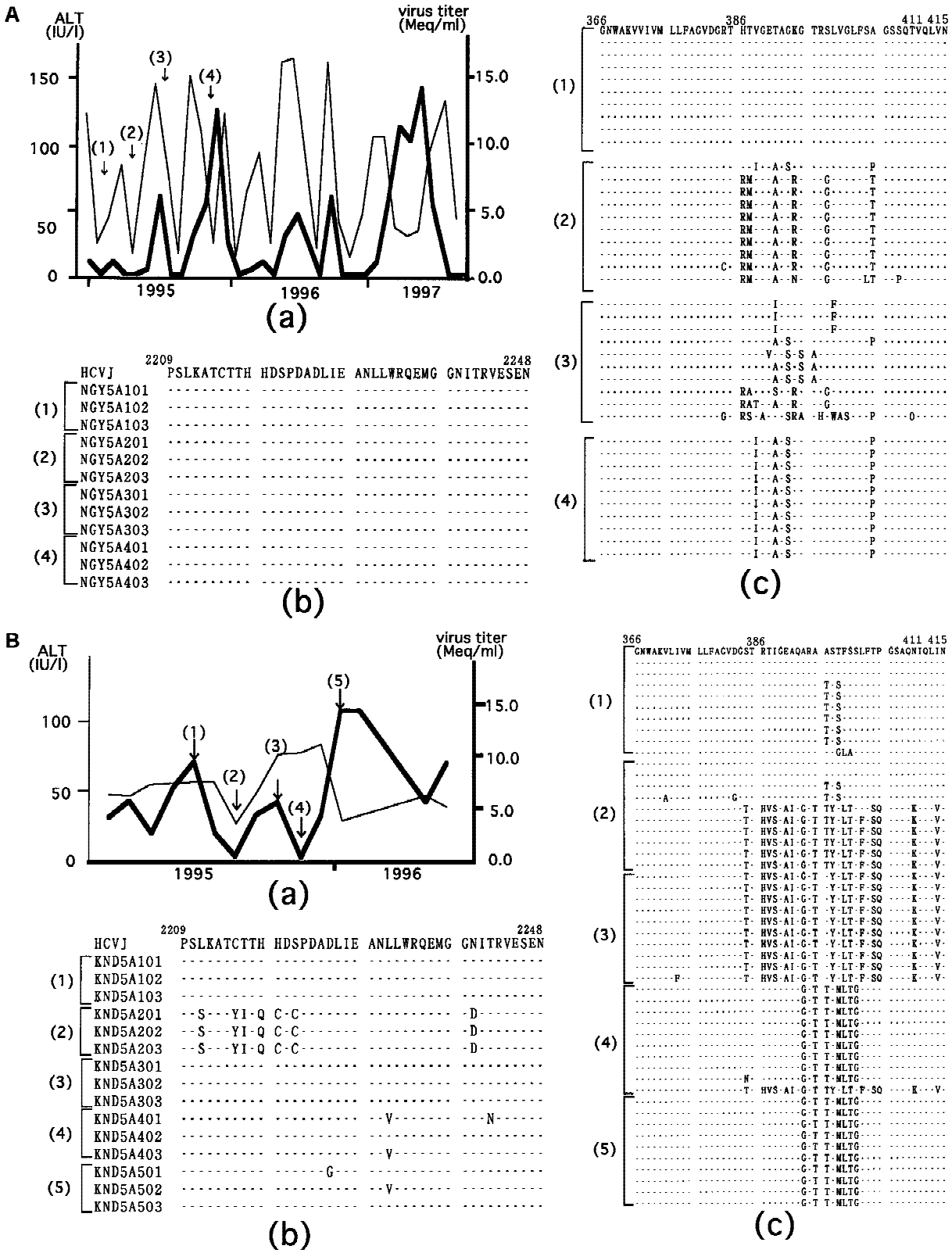
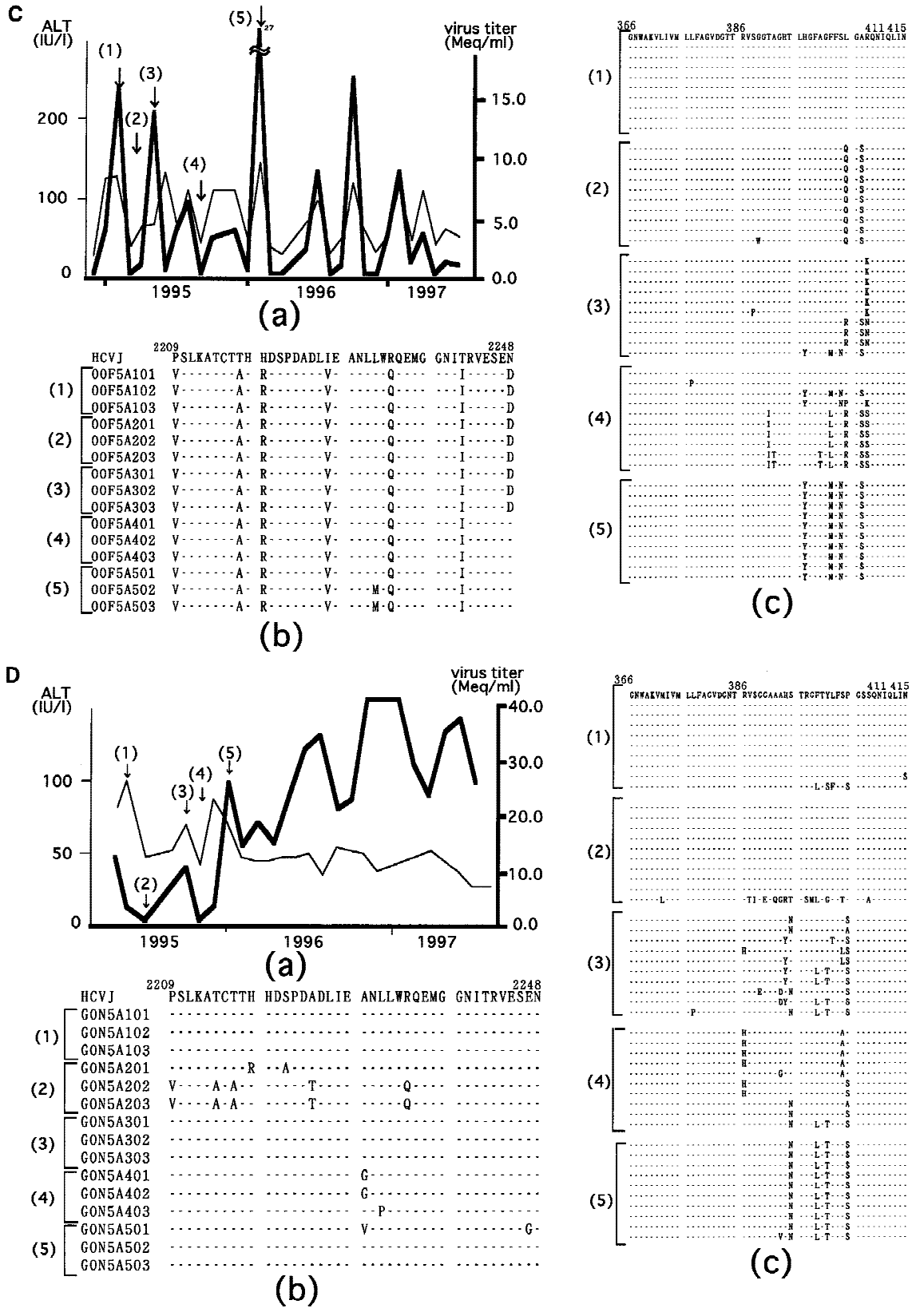


Fig. 2. Serial changes in alanine amino transferase (ALT), hepatitis C virus (HCV) viral titer, and amino acid sequences (by standard single letter codes) in five patients **(A–E)** with marked fluctuation of the virus titer. (a) Serial changes in ALT (thin line) and bDNA concentrations (thick line). (b) Amino acid sequences of the interferon sensitivity determining region (ISDR) compared with prototype HCV genotype 1b (HCVJ [Kato et al., 1990]). The dashes indicated residues identical to amino acid in HCVJ. Sections (1)–(5) represent the time intervals when serum samples were obtained, as indicated by the numbers in parentheses in (a) of A–E. (c) Amino acid sequences of hypervariable region 1 during the same time intervals shown in (a). The upper sequence was the most major sequence at point (1), and the same amino acids are shown as dashes in lower sequences.



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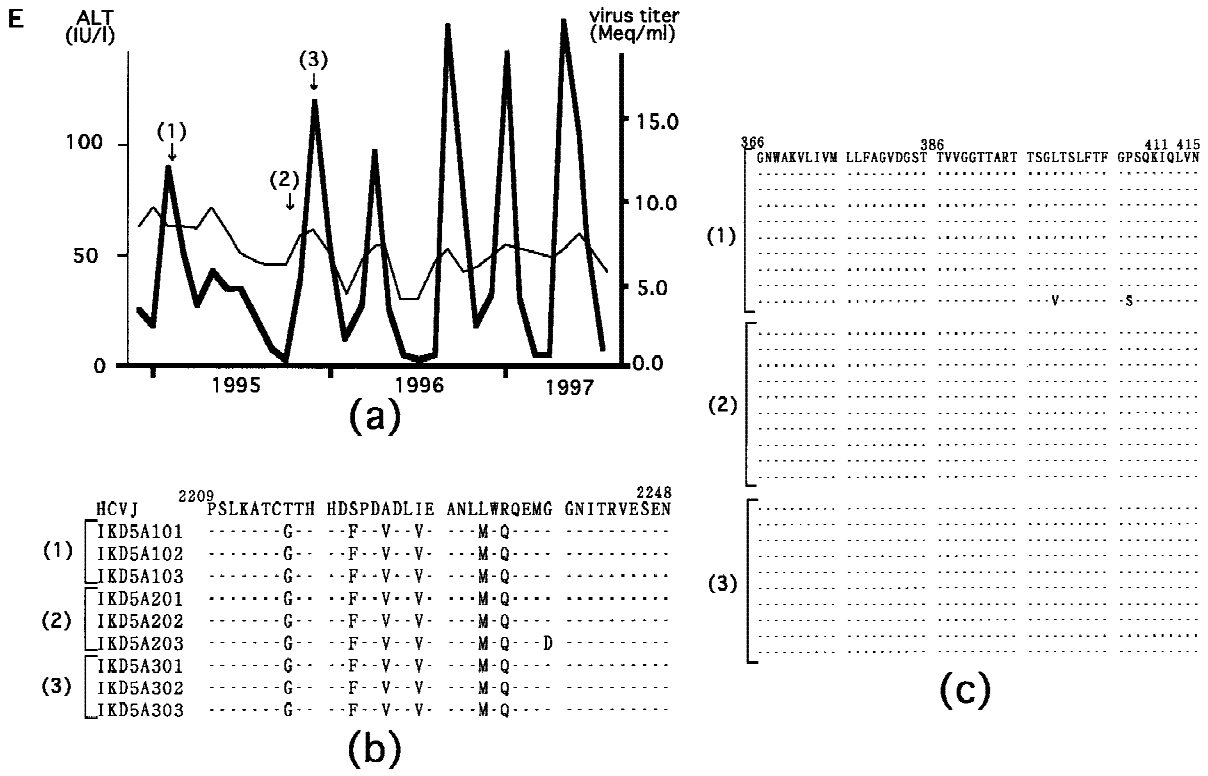

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attempt to identify amino acid substitutions associated with elevation or reduction of viral titers. One of the domains studied, HVR1, comprises part of the envelope protein of HCV. It seems likely that the production of neutralizing antibodies against HVR1 in the envelope E2/NS1 protein [Weiner et al., 1991; Kato et al., 1993] may reduce the viral titer, and that subsequent emergence of "escape mutants" may increase viral load. However, the results showed no apparent relationship between amino acid substitutions in HVR1 and HCV titer. Considering the quasispecies nature of HCV and the rapid virus turnover, it is possible that mutants that escaped antibody neutralization by amino acid substitution of HVR1 rapidly replaced the neutralized strain, and hence no noticeable titer change occurred with the substitutions. In contrast, the virus titers tended to correlate with ALT values in these patients (Fig. 2).

We also analyzed the ISDR region. Previous studies from our laboratory and those of other investigators identified frequent multiple amino acid substitutions in the ISDR in patients with low viral titers [Enomoto et al., 1995, 1996; Chayama et al., 1997]. The present findings are consistent with those results in patients with stable viral titers (Fig. 3). Two recent reports showed amino acid substitutions [Hijikata et al., 1998; Polyak et al., 1998]. Hijikata et al. [1998] reported a "take-over" of the wild type by the mutant type or vice versa in two patients. These patients are similar to the patient shown in Figure 4 with fluctuation of ISDR quasispecies. Although these investigators suggested that immune pressure against virion surface epitopes plays an insidious role for the apparent selection of HCV ISDR quasispecies, the direct evidence for such selection remains to be elucidated. On the other hand, two of three patients described by Polyak et al. [1998] had one amino acid substitution during 11- and 10-year follow up, indicating that the amino acid sequence in this region is relatively stable. However, the single patient with multiple amino acid substitutions during 13-year follow up is remarkable because multiple amino acid substitutions were present despite the relatively high viral load (6.8 and 5.8 Meq/ml). The incidence and characteristics of such rare patients should be investigated further. Although these two studies did not examine the relationship between amino acid substitutions and viral titer, the present results showed that amino acid substitutions actually accompany changes in viral titer (Figs. 2B and D). However, changes in viral titer also occurred without amino acid substitutions in the ISDR (Fig. 2), indicating that amino acid substitutions in this region are not tightly linked to fluctuations in viral titer. On the other hand, ALT activity in patients with marked fluctuations of viral titer correlated with viral load during serial monthly observations (Fig. 2). This finding suggests that fluctuations in virus titer could be explained by immunological clearance, by cytotoxic T lymphocytes, of liver cells that

No.	Amino acid sequence HCVJ PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN	Virus titer	Follow up period (mo)	Number of sample
1	----------	High	24	2
2	----------	High	24	6
3	----------	High	24	7
4	----------	High	20	14
5	----------	High	14	8
6	----------	High	24	2
7	---------R----------	High	24	4
8	---------R----------	High	24	2
9	---------R----------	High	24	8
10	---R----------	High	24	8
11	----------	Internediate	24	6
12	----------	Internediate	24	8
13	----------	Internediate	24	11
14	----------	Internediate	24	10
15	----------	Internediate	24	6
16	----------	Internediate	24	9
17	----------	Internediate	24	4
18	----------	Internediate	17	2
19	----------	Internediate	24	9
20	----------	Internediate	24	5
21	---------R----------	Internediate	24	10
22	---------R----------	Internediate	18	3
23	---------R----------	Internediate	18	3
24	---------R----------	Internediate	18	7
25	---------R----------	Internediate	24	6
26	---------R----------	Internediate	12	4
27	---------N----------	Internediate	24	4
28	---------C----------	Internediate	24	4
29	--------AC----------	Internediate	24	5
30	---------R---L----------	Internediate	24	9
31	---------R----------	Low	24	3
32	V--------R----------K	Low	22	6
33	L----A--AR-----V--V----------	Low	24	5
34	V----A--A-QC---V-------M----------	Low	24	2

Fig. 3. Amino acid sequence analyses of interferon sensitivity determining region (ISDR) of 34 patients with a stable virus titer. Each amino acid sequence was indicated the same as in Figure 2. For categorization of the viral titer, see legend of Figure 1. Follow-up period: the length of observation in months, number of sample: number of serum samples analyzed for amino acid sequences of ISDR.

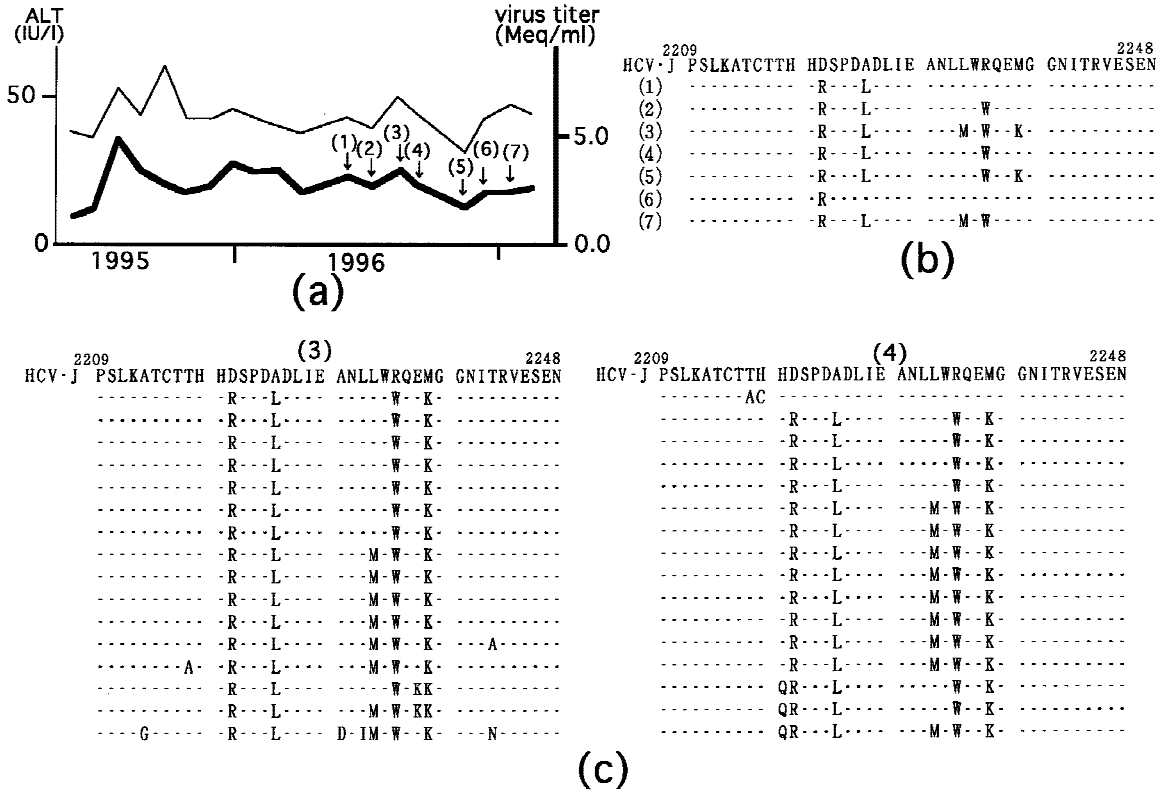

Fig. 4. Serial changes in virus titer and alanine amino transferase (ALT) in a single patient who showed amino acid substitutions during follow-up and amino acid sequences of interferon sensitivity determining region (ISDR). **(a)** Serial changes in ALT (thin line) and bDNA values (thick line). **(b)** Amino acid sequences of ISDR obtained by direct sequencing methods. (1)–(7) in (b) represent the time intervals of analysis shown in (a). **(c)** Amino acid sequences of ISDR obtained after cloning of amplified polymerase chain reaction (PCR) products. Sixteen independent clones of hepatitis C virus obtained at time intervals (3) and (4) in (a) were sequenced. Each amino acid sequence is indicated as in all (b) panels of Figure 2.

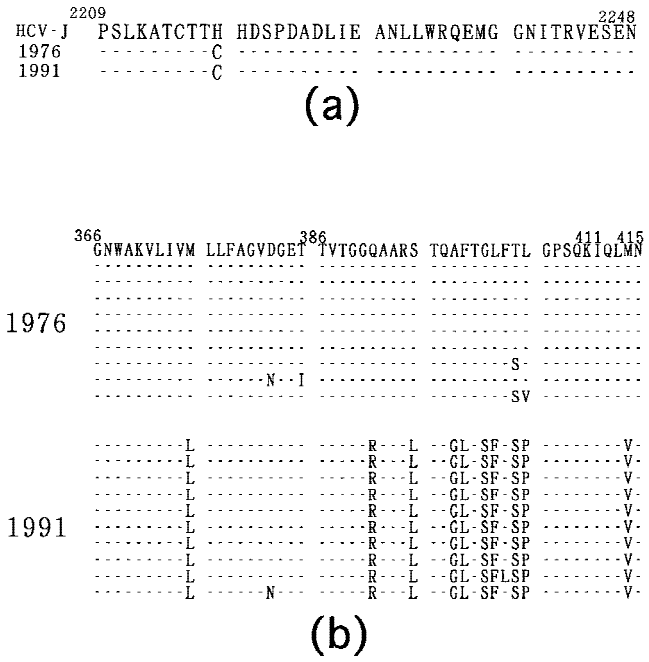

Fig. 5. Amino acid sequences of hepatitis C virus (HCV) of a single patients who was followed-up from 1976 until 1991 and belong to intermediate titer group in HCV titer (Fig. 1). **(a)** Amino acid sequences of interferon sensitivity determining region (ISDR) obtained in 1976 and 1991 by direct sequencing, and indicated as in all (b) panels of Figure 2. **(b)** Amino acid sequences of hypervariable region 1 (HVR1) of serum samples obtained between 1976 and 1991. Ten independent clones were sequenced. The upper sequence was the most major sequence in 1976, and identical amino acids are indicated as dashes in the lower sequences.

have started to harbor active virus replication [Kato et al., 1993; Shirai et al., 1994; Kita et al., 1995; Kaneko et al., 1996; Rehermann et al., 1996; Nelson et al., 1997]. Monthly observations did not provide a clear evidence that increases in ALT occurred immediately after emergence and proliferation of variants with amino acid substitutions in T-cell epitopes. More frequent and extensive amino acid sequence analyses are therefore necessary to elucidate the mechanism of titer fluctuation associated with fluctuations in ALT levels. Evidence that liver cells harboring increased HCV variants are actually killed by activated cytotoxic T cells is also required.

The mechanism of ISDR amino acid substitution accompanying marked reduction in virus titer is unknown. Our data showed that amino acid sequences in this region were stable in all patients during short-term (Fig. 3) and long-term (15-year) observations (Fig. 5). Amino acid substitutions appeared in only two patients with low viral titers (Figs. 2B and D). These observations indicate that mutations in the ISDR may occur under certain circumstances that are unfavorable for viral replication. In duck hepatitis virus, the wild-type virus has been shown to replicate preferentially in ducklings when inoculated as a mixture with a precore mutant [Weiner et al., 1995]. Furthermore, nucleotide sequence substitutions in the core region of hepatitis B virus accumulate along with enhanced host cellular immune responses [Chuang et al., 1993; Akarca and Lok, 1995; Hosono et al., 1995; Asahina et al., 1996; Li et al., 1996]. These mutations were observed in both human leukocyte antigen (HLA) epitopes and other nonepitope portions, suggesting that the virus evades immunological attack by amino acid substitutions in the major HLA epitope, thereby facilitating additional amino acid substitutions. Whether this hypothesis is applicable to HCV requires further investigation.

Although possible mechanisms for HCV to evade the effect of interferon on the ISDR have been presented [Lee et al., 1996], whether the ISDR actually "determines" the effect of interferon is still controversial. The appearance of amino acid substitutions in this region in two of the five patients during low virus load supported the findings of Enomoto et al. [1996] that amino acid substitutions in the ISDR are related to low virus titer and hence determine the sensitivity of the virus to interferon. However, changes in the viral titer also occurred without substitutions in this region (Figs. 2A, C, E). This finding is consistent with previous observations that viral titer and amino acid substitutions in the ISDR are independent predictive factors of the effect of interferon [Chayama et al., 1997]. It may be assumed that during active immunological reaction to HCV, interferon eradicates the virus in concert with the immune system. Further studies are necessary to elucidate the mechanisms of association of amino acid substitutions in the ISDR with effects of interferon to establish more effective strategies for eradication of HCV by interferon and other antiviral drugs.

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